Docket Number: 62942-A-PRO/JPW/SHS

Application for United States Letters Patent

To all whom it may concern:

Be it known that We, William C. Olson and Paul J. Maddon

have invented certain new and useful improvements in COMPOSITIONS AND METHODS FOR INHIBITION OF HIV-1 INFECTION

of which the following is a full, clear and exact description.

Applicants: Olson and Maddon
Serial No. : 09/594,938
Filed: June 15, 2000
Exhibit 16

Mechanisms of Synergy Between HIV-1 Attachment, Coreceptor and Fusion Inhbitors

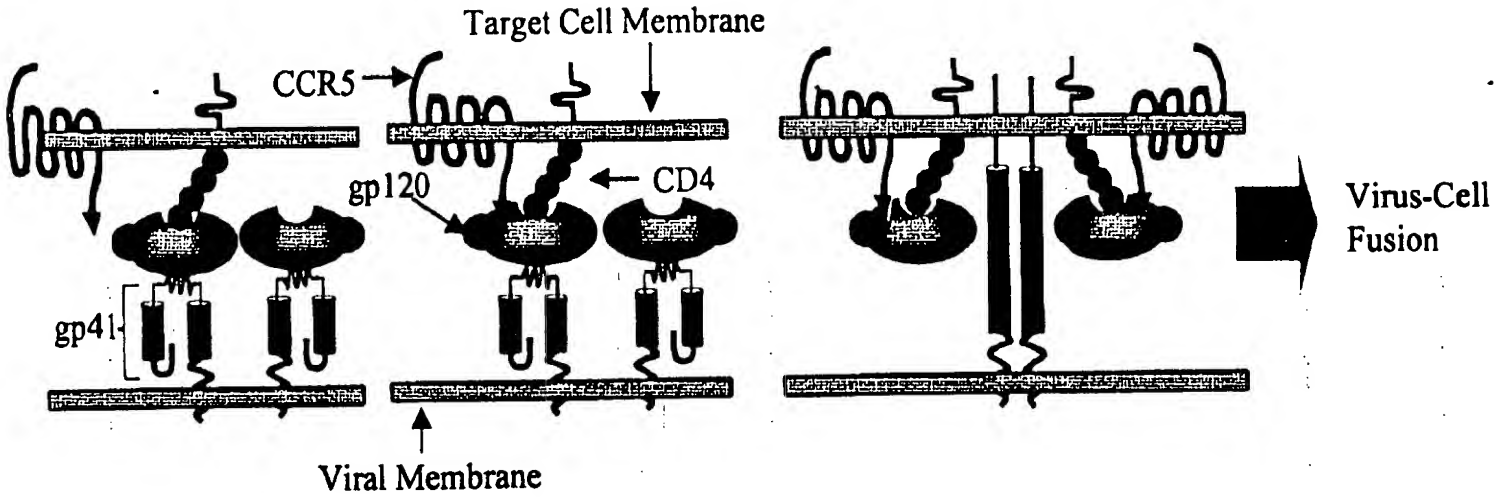
#310

Abstract

HIV-1 entry proceeds via a cascade of events that provide promising targets for a new generation of antiviral therapies, including the gp120-CD4 attachment inhibitor PRO 542, the gp120-coreceptor inhibitor PRO 140, and the gp41 fusion inhibitors T-20 and T-1249. The multi-step nature of HIV-1 entry leaves the virus highly susceptible to inhibition by combinations of drugs that act at distinct stages of this process. We have shown that double- and triple-drug cocktails of attachment, coreceptor, and the fusion inhibitors potently and synergistically block HIV-1 entry over a wide range of experimental conditions *in vitro*.

The present study was performed in order to identify the molecular basis of the observed synergistic interactions. HIV-1 membrane fusion was monitored in real time using a semi-automated fluorometric assay, and the inhibitory activities of the individual drugs and drug cocktails were evaluated in time-of-addition, washout, and stepwise temperature-controlled studies. The data were consistent with a model wherein the drugs act cooperatively to delay the recruitment of a critical number of fusion-active HIV-1 envelope glycoproteins to the site of the fusion pore. These findings have important implications for the combination use of HIV-1 entry inhibitors *in vivo*, which may emerge as an important new paradigm for antiviral therapy.

HIV-1 Entry Involves at Least Three Steps that Provide Promising Targets for Therapy



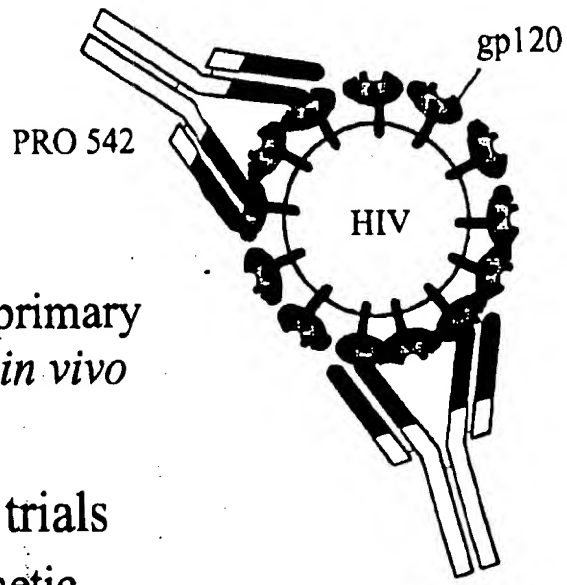
gp120-CD4 attachment

gp120-coreceptor interactions

gp41-mediated fusion

PRO 542 (CD4-IgG2)

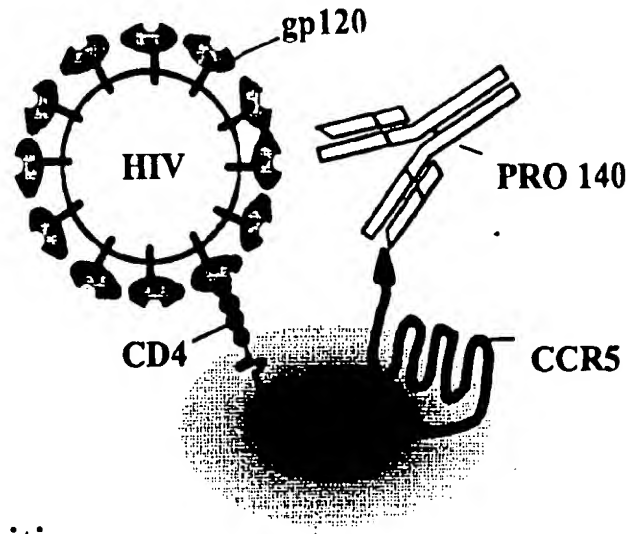
attachment inhibitor



- Tetravalent CD4-based protein
  - Broadly and potently neutralizes primary HIV isolates *in vitro*, *ex vivo* and *in vivo*
  - Acts prior to fusion
- Completed two Phase I/II clinical trials
  - Excellent safety and pharmacokinetic profiles, non-immunogenic
  - Statistically significant reductions in viral load with a single dose
  - Sustained reductions in free and cell-associated infectious virus
- In Phase II testing

PRO 140

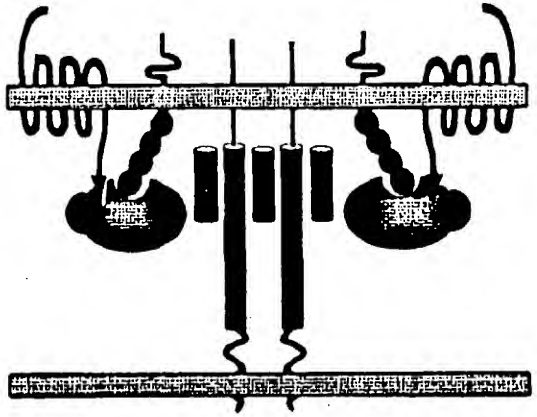
coreceptor inhibitor



- Monoclonal antibody to CCR5
- Potently neutralizes CCR5-using viruses
  - protects primary T cells and macrophages
  - genetic subtype-independent inhibition
- Selectively blocks HIV entry without affecting chemokine-induced signaling
- No emergence of HIV resistance following 31 weeks *in vitro* culture
- Phase I trials scheduled for 2001

T-20

fusion inhibitor

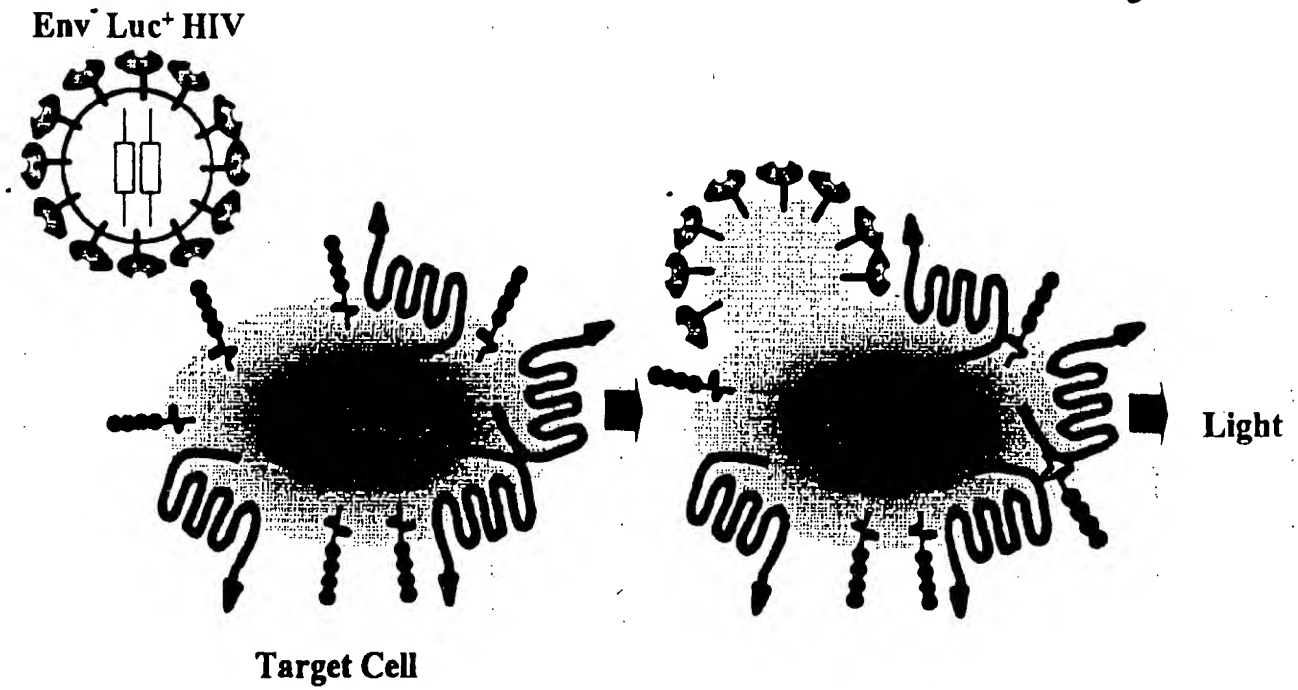


- Developed by Trimeris, Inc. in collaboration with F. Hoffmann-La Roche
- Peptide derived from HIV-1 gp41
- Broadly and potently blocks HIV-1 cell-cell and virus-cell fusion *in vitro*
- Completed Phase I/II clinical trials
  - Good tolerability
  - pK supports twice daily subcutaneous dosing
  - Clinically significant dose-dependent reductions in viral load
  - In Phase III testing

Combination Use of Entry Inhibitors

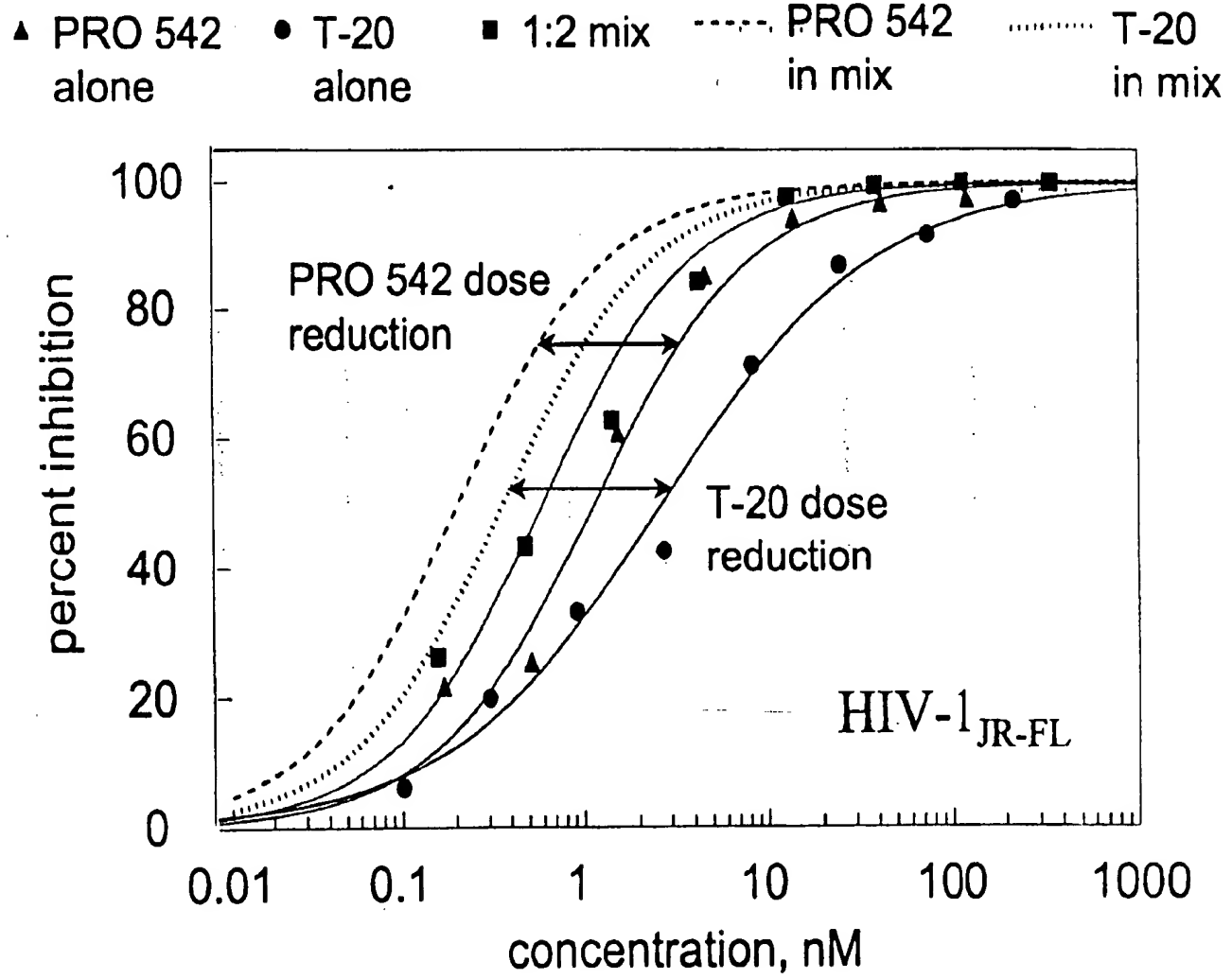
- Combination therapy is required to control HIV *in vivo*
- HIV entry inhibitors may represent the next major advance in therapy
- Critical to determine drug-drug interactions earlier rather than later in clinical development
  - avoid antagonistic combinations
  - identify synergistic combinations
- Clinical benefits of synergistic combinations
  - more potent antiviral effects
  - more durable antiviral effects

HIV-1 Virus-Cell Fusion Assay



Single-cycle HIV-1 entry assay. Env⁻Luc⁺ HIV reporter viruses were prepared as described (Dragic *et al.*, Nature, 381:667) . The viruses are complemented with gp120/gp41 from an HIV strain of interest and thus are capable of fusing with target cells that express CD4 and fusion coreceptors, resulting in the introduction of the viral genes into the cell. Because the viruses encode the luciferase reporter enzyme rather than HIV env, infection leads to the production of luciferase rather than new viral particles. The amount of luciferase is measured 72 hr post-infection using standard methods.

Synergistic Inhibition of Virus-Cell Fusion with PRO 542 and T-20 (I)

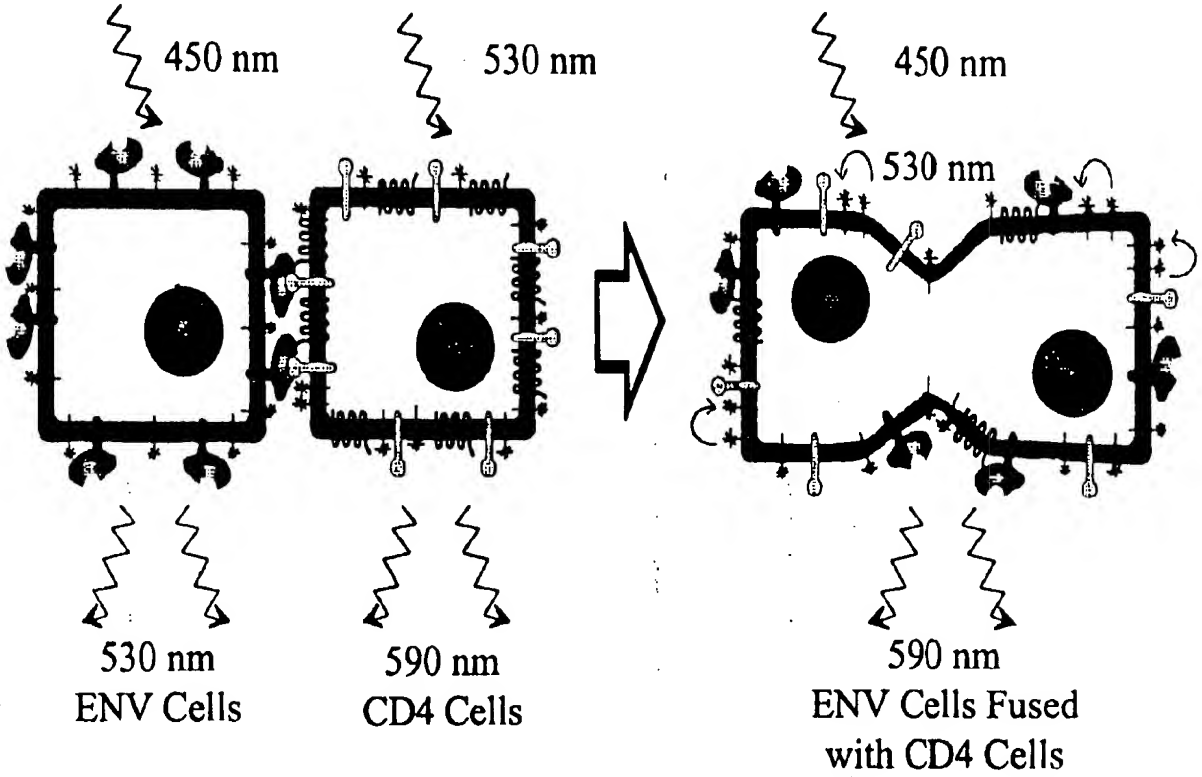


Synergistic Inhibition of HIV-1 Virus-Cell Fusion with PRO 542 and T-20 (II)

	Percent Inhibition	Combination Index	Inhibitory Conc., nM		Dose Reduction	
			PRO 542	T-20	PRO 542	T-20
JR-FL (R5)	95	0.14	30	120	11	24
	90	0.18	12	45	8.0	17
	70	0.29	2.5	8.0	5.7	10
	50	0.39	0.92	2.7	4.4	7.3
DH123 (R5X4)	95	0.36	65	123	5.9	6.2
	90	0.45	20	54	4.0	6.1
	70	0.76	2.4	12	2.0	5.7
	50	1.1	0.64	4.8	1.3	5.5

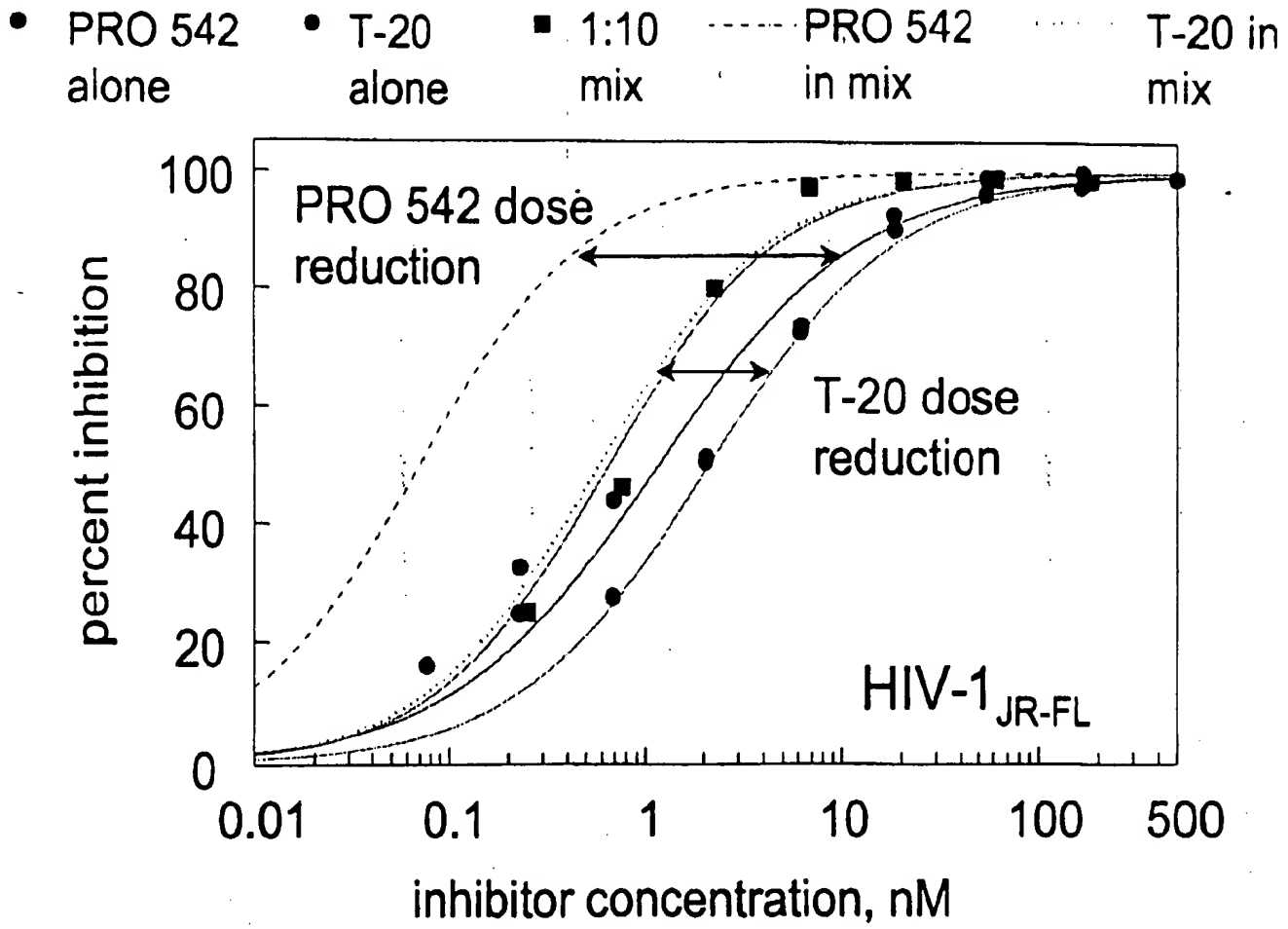
PRO 542 and T-20 were used in a 1:2 molar ratio

HIV-1 Cell-Cell Fusion Assay



Resonance Energy Transfer (RET) assay of HIV-1 membrane fusion. Cells expressing gp120/gp41 (ENV cells) are labeled with the fluorescent dye octadecyl fluorescein (F18), while T cells that express CD4 and fusion coreceptors (CD4 cells) are labeled with octadecyl rhodamine (R18). The emission spectrum of F18 overlaps with the excitation spectrum of R18, allowing fluorescence RET to occur when the dyes are placed in the same membrane following fusion. RET is measured by stimulating F18 at 450 nm and measuring the R18 emission at 590 nm. The RET signal is directly related to the amount of HIV-1 fusion (Litwin *et al.*, J. Virol. 70:6437)

Synergistic Inhibition of Cell-Cell Fusion with PRO 542 and T-20 (I)

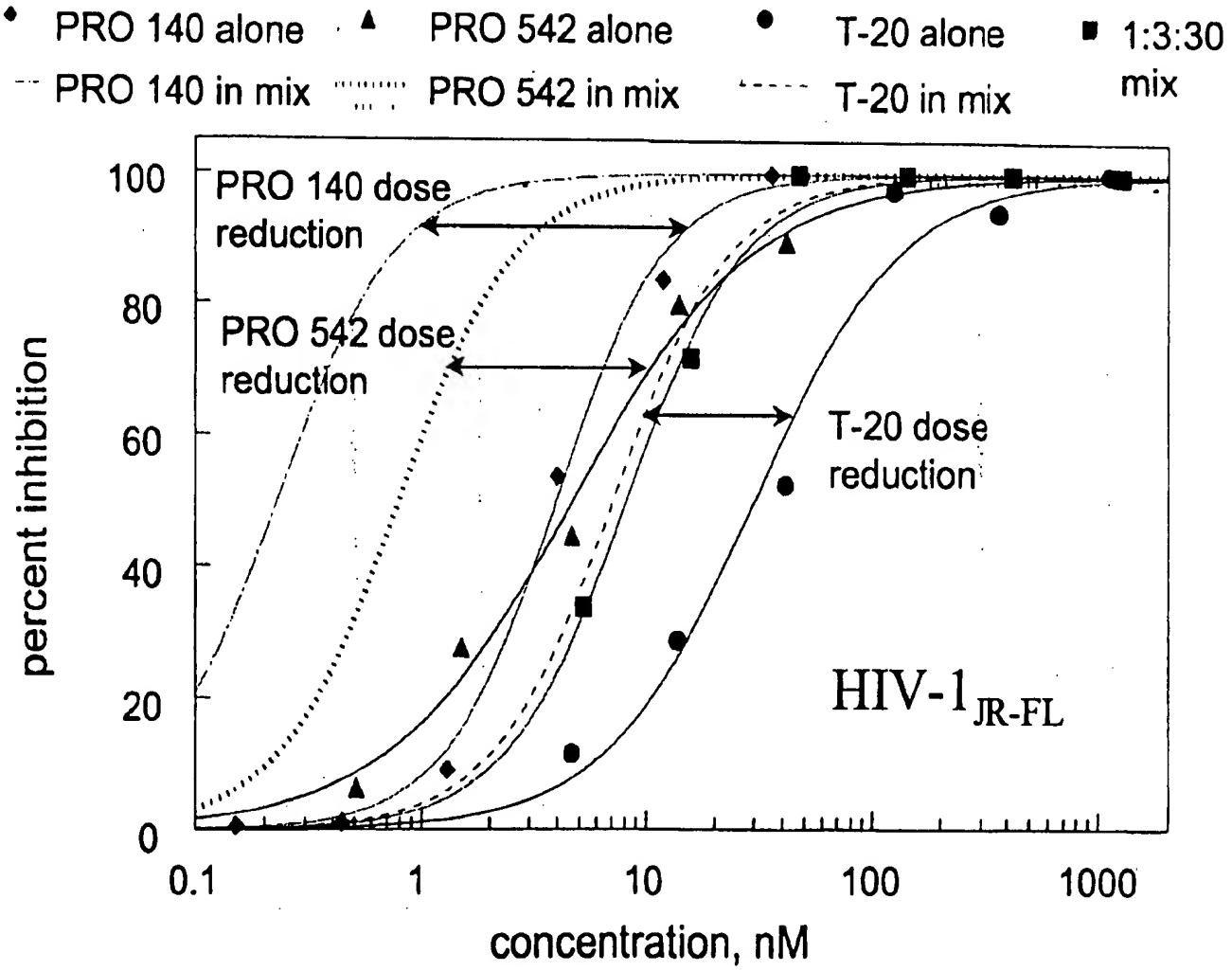


Synergistic Inhibition of HIV-1 Cell-Cell Fusion with PRO 542 and T-20 (II)

Conc. Ratio	Percent Inhibition	Combination Index	Inhibitory Conc, nM		Dose Reduction (fold)	
			PRO 542	T-20	PRO 542	T-20
1:2	95	0.32	95	47	17	4.9
	90	0.38	39	22	13	4.2
	50	0.69	3.0	2.5	6.2	2.8
1:10	95	0.27	28	58	20	4.8
	90	0.28	11	22	20	4.5
	50	0.34	0.84	1.3	22	3.7
1:50	95	0.33	47	120	56	3.2
	90	0.34	15	42	50	3.2
	50	0.38	0.49	1.8	35	3.0

Virus: $HIV\text{-}1_{JR\text{-}FL}$

PRO 140, PRO 542 and T-20 Triple Combination Synergistically Blocks HIV-1 Entry (I)

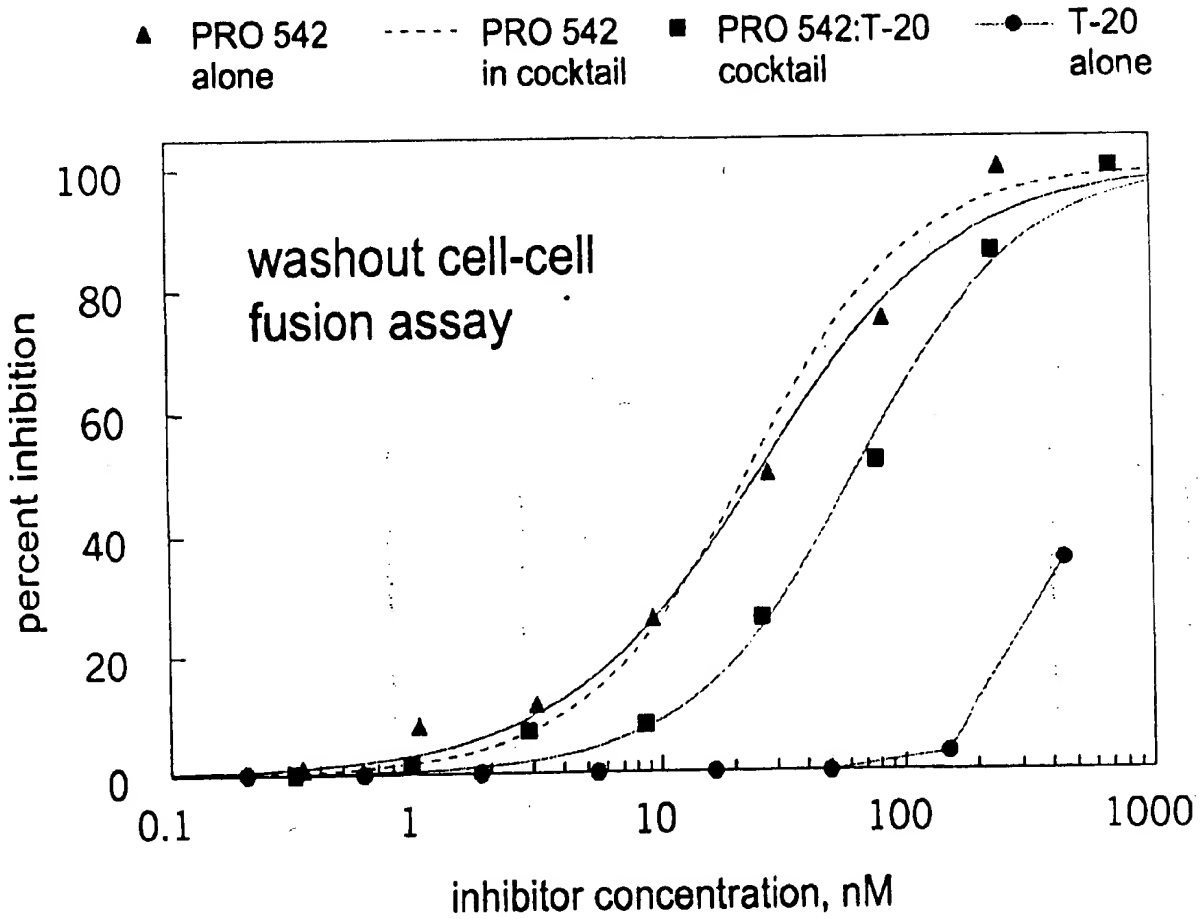


PRO 140, PRO 542, T-20 Triple Combination Synergistically Blocks HIV-1 Entry (II)

Percent Inhibition	Combination Index	Inhibitory Conc, nM			Dose Reduction (fold)		
		PRO 140	PRO 542	T-20	PRO 140	PRO 542	T-20
95	0.24	24	61	160	17	12	7.1
90	0.22	23	32	96	21	8.4	7.4
70	0.19	20	9.8	40	32	4.5	8.9
50	0.18	18	4.7	23	41	3.0	10

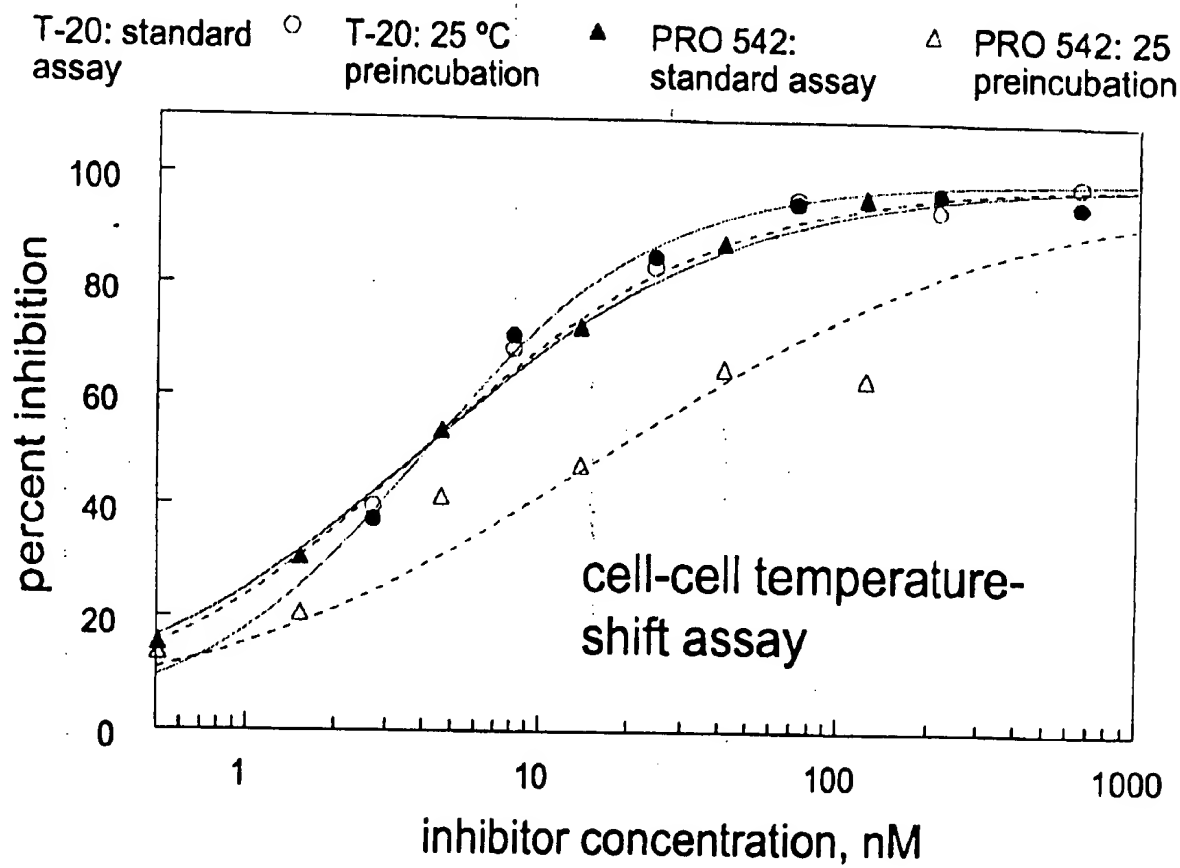
Inhibition of HIV-1_{JR-FL} mediated cell-cell fusion with PRO 140, PRO 542 and T-20 used in a 1:3:30 molar ratio.

PRO 542 Does Not Potentiate T-20 Activity in the Absence of Coreceptor



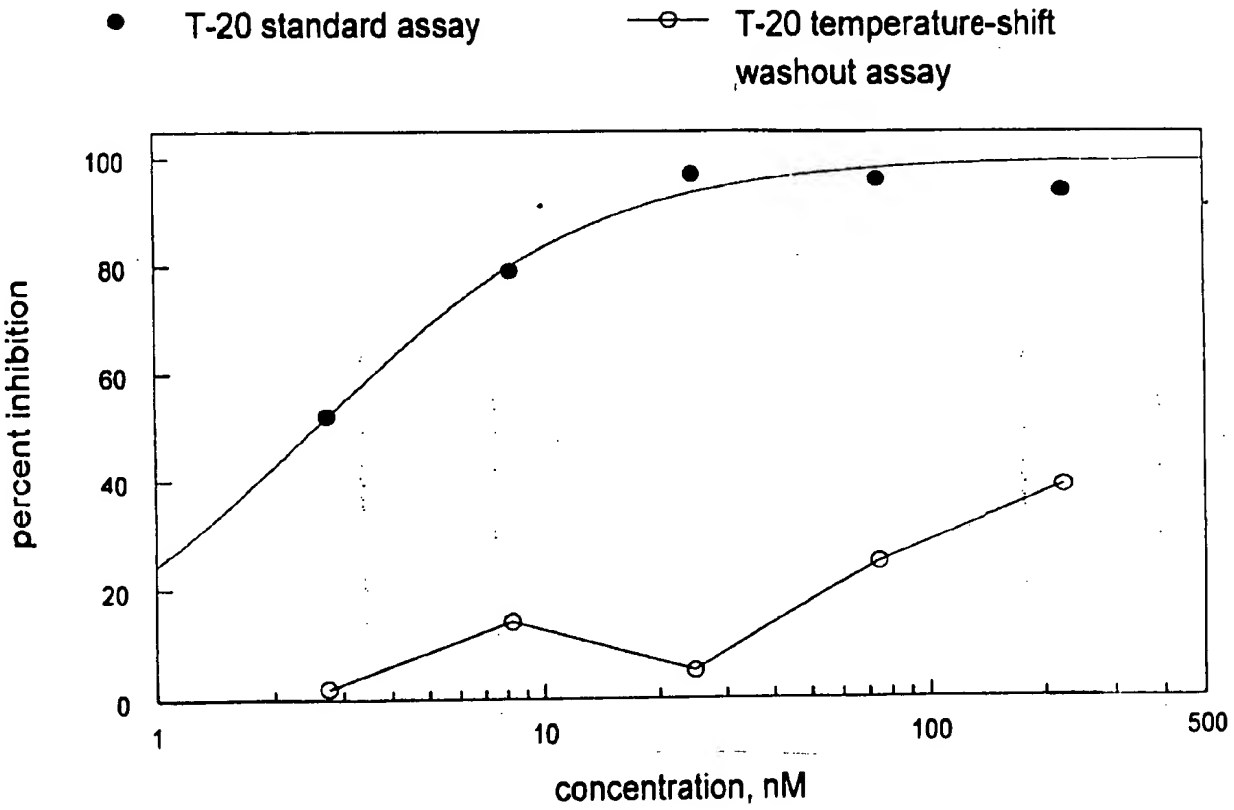
Methods: PRO 542, T-20 or a 1:2 mixture was pre-incubated with HeLa-$ENV_{JR\text{-}FL}$ cells for 2h at 37°C prior to washing, addition of PM1 target cells, and completion of the fusion assay.

Formation of the Prehairpin Intermediate Requires CD4, Coreceptor and 37 °C (I)



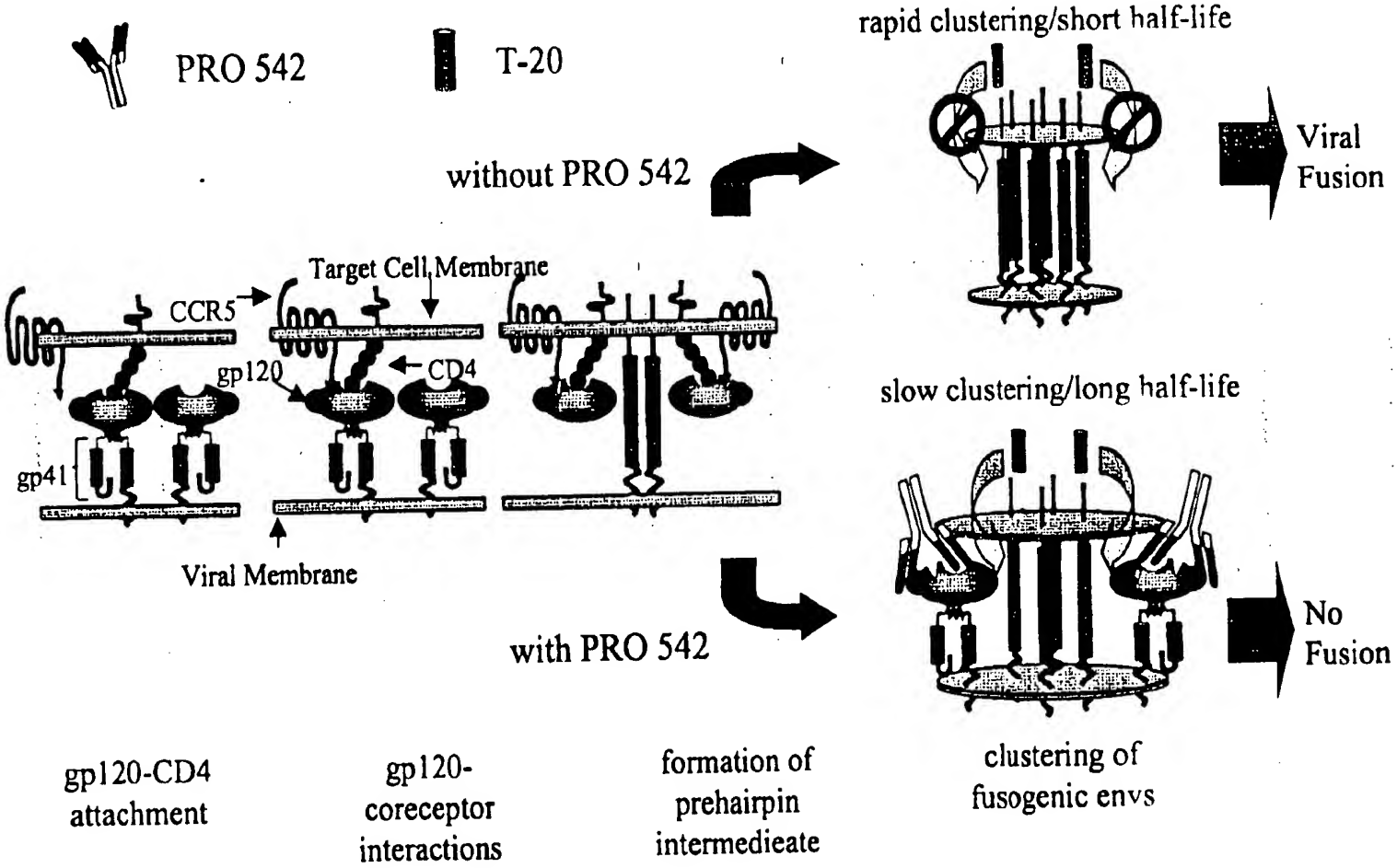
Methods: HeLa-ENV$_{JR\text{-}FL}$ and PM1 target cells were pre-incubated for 2h at 25 °C prior to the addition of drug and warming to 37 °C. Data for standard assays (without preincubation) are shown for comparison.

Formation of the Prehairpin Intermediate Requires CD4, Coreceptor and 37 °C (II)



Methods: HeLa-ENV_{JR-FL} and PM1 target cells were pre-incubated for 2h at 25 °C prior to the addition of drug and warming to 37 °C. Data for standard assays (without preincubation) are shown for comparison.

Possible Mechanism of Synergy: PRO 542 Increases the Half-Life of the T-20 Targets



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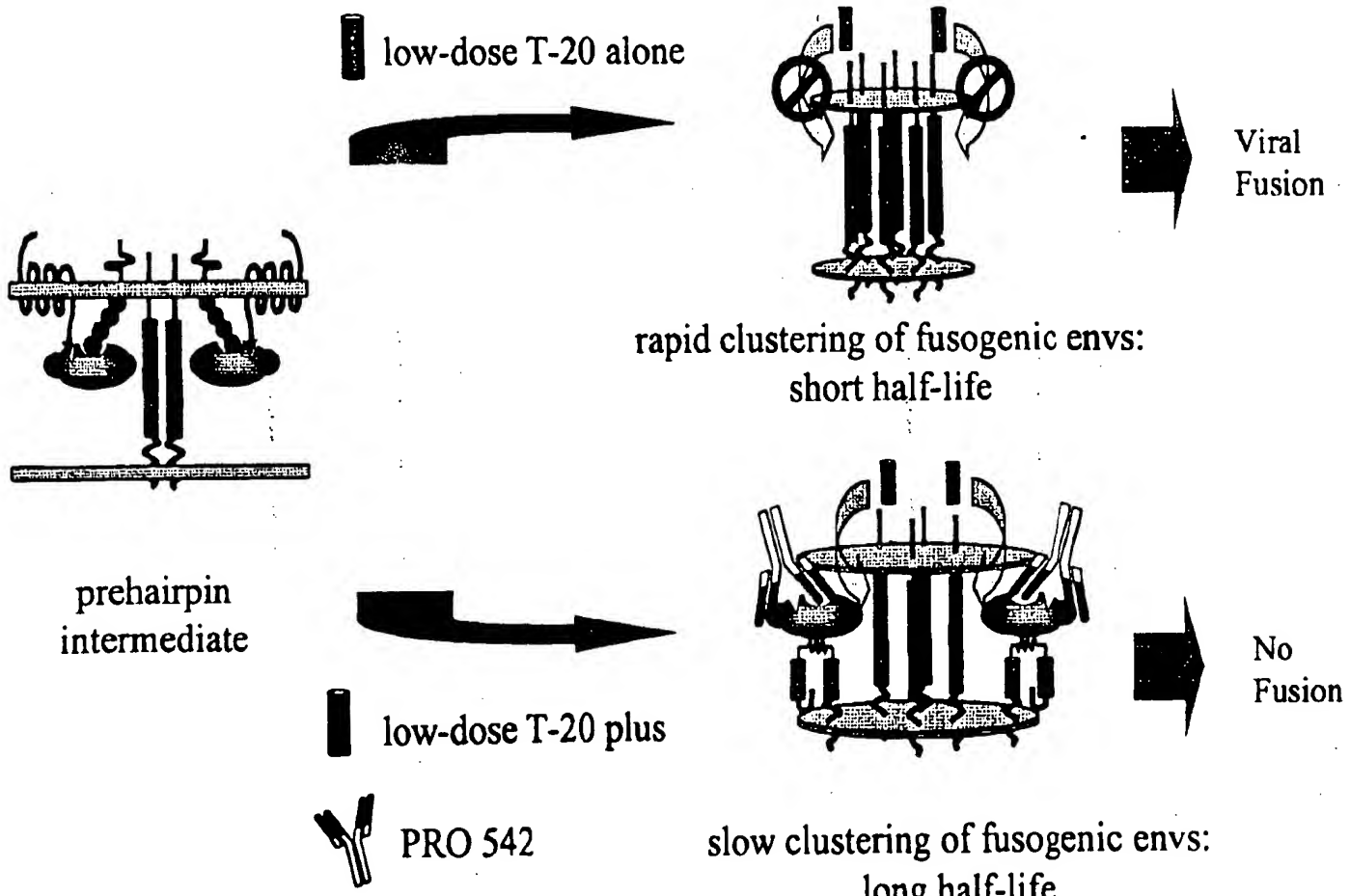

Conclusions

- Double and triple combinations of PRO 542, T-20 and PRO 140 potently and synergistically block HIV-1 entry
- Potent synergies are observed over a broad range of conditions
  - varying drug concentrations
  - varying drug concentration ratios
  - diverse viruses
  - virus-cell and cell-cell fusion
- Synergies are maximal at high levels of inhibition
- PRO 542 does not induce formation of a fusogenic prehairpin intermedidate
- Animal studies are in progress
- The findings provide a compelling rationale for human clinical trials exploring combinations of entry inhibitors for HIV therapy